

SDF-1/CXCL12 Production by Mature Dendritic Cells Inhibits the Propagation of X4-Tropic HIV-1 Isolates at the Dendritic Cell–T-Cell Infectious Synapse[∇]

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An efficient mode of HIV-1 infection of CD4 lymphocytes occurs in the context of infectious synapses, where dendritic cells (DCs) enhance HIV-1 transmission to lymphocytes. Emergence of CXCR4-using (X4) HIV-1 strains occurs late in the course of HIV-1 infection, suggesting that a selective pressure suppresses the switch from CCR5 (R5) to X4 tropism. We postulated that SDF-1/CXCL12 chemokine production by DCs could be involved in this process. We observed CXCL12 expression by DCs *in vivo* in the parafollicular compartment of lymph nodes. The role of mature monocyte-derived dendritic cells (mMDDCs) in transmitting R5 and X4 HIV-1 strains to autologous lymphocytes was studied using an *in vitro* infection system. Using this model, we observed a strong enhancement of lymphocyte infection with R5, but not with X4, viruses. This lack of DC-mediated enhancement in the propagation of X4 viruses was proportional to CXCL12 production by mMDDCs. When CXCL12 activity was inhibited with specific neutralizing antibodies or small interfering RNAs (siRNAs), the block to mMDDC transfer of X4 viruses to lymphocytes was removed. These results suggest that CXCL12 production by DCs resident in lymph nodes represents an antiviral mechanism in the context of the infectious synapse that could account for the delayed appearance of X4 viruses.

HIV-1 strains that use CCR5 for entry (R5 strains) are responsible for most transmission events and predominate in both early and chronic phases of infection (36, 37), while later stages of disease are characterized by the frequent emergence of variants that use both CCR5 and CXCR4 (R5X4 dual-tropic strains) or CXCR4 alone (X4 strains). About half of the individuals infected with B clade HIV-1 switch coreceptor use from CCR5 to CXCR4, and the emergence of X4 viruses is associated with accelerated CD4⁺ T-cell decline and fast progression to AIDS (40). The R5-to-X4 switch is associated with mutations in residues located within the V3 region of gp120, which tend to increase the overall positive charge of the V3 loop (15). Because only a limited number of mutations are required for this phenotypic switch (38, 46), the emergence of X4 variants would be expected to take place on multiple occasions throughout infection. Furthermore, there is evidence that X4 HIV-1 strains are present as minor viral populations in patients in whom R5 HIV-1 isolates predominate (11), and the fast emergence of X4 HIV-1 isolates following treatment with potent CCR5 antagonists (47) extends that observation. Moreover, CXCR4 expression is more widespread than CCR5 expression (5, 6). Thus, the failure of X4 HIV-1 to expand during natural infection is an apparent paradox suggesting the pres-

ence of selective pressures influencing tropism evolution, but the mechanisms governing such selection are not fully understood.

Myeloid and plasmacytoid dendritic cells (PDCs) represent the two main subsets of DCs that have been described in humans. Despite sharing common antigens, their functions and roles in HIV-1 infection are radically different. DCs are the most potent antigen-presenting cells *in vivo* (4, 44). Immature DCs (iDCs) migrate specifically to sites of inflammation to capture pathogens and pathogen-associated antigens, which are processed into antigenic peptides and presented on major histocompatibility complex class II molecules. Once activated by pathogen encounters, DCs mature and migrate to the T-cell areas of secondary lymphoid organs, where they interact with and activate resting T cells and initiate adaptive immune responses (4, 27). PDCs are located in blood and secondary lymphoid organs, but they can be recruited to sites of inflammation and are thought to play an important role in innate immune responses to different types of viruses by producing alpha interferon (IFN- α).

Certain subsets of DCs residing in the peripheral mucosae are the first immunocompetent cells to encounter lentiviruses (21, 39). Successful infection of a host by HIV-1 requires the dissemination of virus from sites of initial infection at mucosal surfaces to T-cell zones in secondary lymphoid organs, where myeloid DCs enhance the infection of CD4⁺ T cells by HIV-1 (10, 33, 34). On the other hand, PDCs inhibit HIV-1 replication in T cells by secretion of IFN- α and yet-unidentified soluble factors (19). The molecular basis underlying DC–T-cell spread of HIV-1 remained unclear until the C-type lectin DC-

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SIGN (DC specific ICAM-3-grabbing nonintegrin) (18) was identified. DC-SIGN is highly expressed on DCs present in mucosal tissues and binds to virus via interaction with the HIV-1 envelope glycoprotein gp120. DC-SIGN efficiently captures HIV-1 virions in the periphery and facilitates their transport to secondary lymphoid organs rich in T cells. DCs facilitate efficient spread of virus to surrounding permissive T cells either by infection *in trans*, in which the DCs present infectious virus to T cells but are not themselves infected (3, 17, 23), or *in cis*, in which the DCs are themselves infected (9, 24).

DCs, macrophages, and intestinal T lymphocytes represent the primary target cell types during mucosal HIV-1 transmission. In these cells, CXCR4 is selectively downmodulated (1, 49), because the CXCR4 ligand stromal-cell-derived factor-1 (SDF-1/CXCL12) is constitutively expressed by epithelial cells within the rectum, endocervix, and vagina (1). Moreover, it has been reported that intestinal epithelial cells transfer R5 viruses, but not X4 viruses, to target cells (28). Such mechanisms may provide a partial explanation for the selection of R5 HIV-1 in the first few days of the infection process during sexual transmission. However, it is still unclear which processes drive the predominance of R5 variants early in the course of parenteral infection and why the emergence of X4 strains, independently of the route of transmission, occurs late in the course of HIV-1 infection.

We postulate that CXCL12 production by DCs in lymph nodes could be involved in these processes. Through this mechanism, CXCL12 could contribute to the selection of R5 viruses during dissemination in secondary lymphoid organs after mucosal and intravenous transmission and to the generally delayed emergence of X4 isolates. To address this hypothesis, we analyzed the impact of CXCL12 on the propagation of X4 and R5 HIV-1 isolates in an *in vitro* model of HIV infection, using an autologous coculture of activated T lymphocytes and monocyte-derived dendritic cells (MDDCs).

MATERIALS AND METHODS

Ethics. The research performed and management of clinical samples were approved by the Bioethical Committee of Instituto de Salud Carlos III. When samples from patients were used to amplify and clone HIV-1 envelopes, informed consent was obtained.

Reagents and cytokines. Interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from R&D Systems and used at 20 ng/ml. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 was obtained from Sigma and used at 100 ng/ml. Phytohemagglutinin (PHA) and IL-2 were purchased from Sigma and Chiron, respectively.

Antibodies. The anti-CXCL12 monoclonal antibody (MAb) K15C [IgG2a(κ)] was generated by immunizing BALB/c mice with the CXCL12-derived peptide KPVLSYRSPSRFFC conjugated via cysteine 15 to bovine serum albumin (BSA) (2). K15C and IgG2a (Becton Dickinson) were used at a final concentration of 30 μg/ml. The rabbit antisera against HIV-1 Gag p17/p24 were obtained from the Centers for AIDS Research (CFAR).

For flow cytometry, CD14, CD83, and CXCR4 were detected using phycoerythrin-conjugated MAbs from clones M5E2, HB15e, and 12G5 (BD Biosciences), respectively. CCR5 was detected using fluorescein isothiocyanate-conjugated MAb (clone 2D7; BD Biosciences). For intracellular staining of CXCL12, cells fixed and permeabilized with 1% paraformaldehyde and 1% Tween 20 were indirectly stained with the anti-CXCL12 K15C antibody. After being stained, the cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (BD Biosciences).

Cells and cell lines. Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy individuals and were purified using Ficoll-Hypaque density centrifugation (BioWhittaker). Adherent cells were depleted of lymphocytes by a 1-h plastic adhesion step at 37°C, followed by extensive washing

TABLE 1. Human CXCL12 cDNA target sequences for siRNAs^a

siRNA	Position	Sequence (5' to 3')
siCXCL12 94	94–112	ACGCCAAGGTCGTGGTCGT
siCXCL12 183	183–201	TGCCGATTCTTCGAAAGCC
siCXCL12 244	244–262	CTCCAAACTGTGCCCTTCA

^a Three different siRNAs directed against human CXCL12 were designed to correspond to distinct parts of the CXCL12 mRNA sequence. Target sequences of the CXCL12 cDNA are shown.

in prewarmed culture medium. Supernatants containing lymphocytes were collected, and the lymphocytes were grown in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) in the presence of IL-2 (300 IU/ml) and PHA (5 μg/ml) for 2 days and IL-2 for an additional 5 days. At the time of coculturing with MDDCs, lymphocytes expressed higher levels of CXCR4 (80 to 90% positive) than of CCR5 (20 to 30% positive). To generate immature MDDCs (iMDDCs), purified monocytes were cultured in the presence of IL-4 (20 ng/ml) and GM-CSF (20 ng/ml) for 7 days. At day 5, mature MDDCs (mMDDCs) were generated by the addition of LPS (100 ng/ml) for 2 days. At day 7, the phenotype of cultured MDDCs was confirmed by flow cytometric analysis. mMDDCs expressed high levels of CD83 (70 to 100% positive) and low levels of CD14 (1 to 5% positive).

293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) (BioWhittaker) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM).

The human T-cell lines Jurkat and MT-2 were grown in RPMI 1640 medium (BioWhittaker) supplemented with 10% inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM).

Plasmids. pNL4-3Luc and pNL4-3Ren carry a full-length HIV-1 genome and the reporter firefly luciferase or *Renilla* luciferase gene, respectively, cloned into the *nef* open reading frame (16). The pJRLuc and pJRRen plasmids were generated by cloning gp160 from the JR-CSF clone (R5 tropism) in place of the NL4-3 envelope in the plasmid pNL4-3Luc or pNL4-3Ren (N. González, M. Pérez-Olmeda, J. García-Pérez, S. Sánchez-Palomino, E. Mateos, S. Spijkers, A. Cascajero, A. Alvarez, and J. Alcamí, unpublished data). pNP1525Ren was constructed by replacing the NL4-3 envelope with gp160 from the reference isolate NP1525 (X4 tropism) in the plasmid pNL4-3Ren. Plasmids carrying *env* from HIV-infected patients were generated by replacing the *lacZ* gene of the pNL-lacZ/*env*-Ren vector with gp160 amplified from patients' plasma. The R5 viruses 11525 and 15214 harbor envelopes from patients with early infection, and the X4 strains 1116, 5006, and 392 harbor envelopes from HIV-infected patients at an advanced stage of the disease. The pNL-lacZ/*env*-Ren vector was generated by cloning the *Renilla* luciferase gene in place of *nef* and replacing the *env* coding sequence with the amino-terminal fragment of the *lacZ* gene (González et al., unpublished). pmxGFP (Amara) encodes the green fluorescent protein (GFP) from the copepod *Pontellina plumata*. The pSPAX2 HIV packaging construct includes the HIV *gag* and *pol* genes (12). pTRIP-Sym-ΔU3-MC1 is an HIV-based lentiviral vector and was provided by P. Charneau (Institut Pasteur, Paris, France). pcDNA3-VSVG encodes the (VSVG) vesicular stomatitis virus G protein.

Vector construction and siRNAs. The pSUPER construct was described previously (7). Oligonucleotides expressing small interfering RNA (siRNA) oligonucleotides directed against human CXCL12 mRNA (Table 1) were designed according to the method of Elbashir et al. (13) and purchased from Sigma. These oligonucleotides were annealed and ligated into pSUPER downstream of the H1 promoter, giving rise to pSUPER-siCXCL12 constructs.

The pTRIP-siCXCL12 constructs were generated by digesting the H1-siRNA cassette from pSUPER-siCXCL12 with XbaI and SalI and inserting it into the NheI and SalI sites in the plasmid pTRIP-Sym-ΔU3-MC1.

mMDDC transfection with pSUPER-siCXCL12 constructs. mMDDCs (5 × 10⁶) were transfected with 5 μg of pSUPER control plasmid or pSUPER-siCXCL12 constructs with a human dendritic cell nucleofector kit (Amara) according to the manufacturer's protocol. To test the efficiency of the transfection, mMDDCs were also transfected with 5 μg of the control plasmid, pmxGFP, and 24 h later, flow cytometric analysis of GFP expression was performed.

Real-time quantitative RT-PCR. Total RNA from MT-2 cells infected with lentiviruses was extracted with an RNeasy Mini Kit (Qiagen), and contaminating genomic DNA was removed by incubation with an RNase-Free DNase Set (Qiagen). Total RNA (1 μg) was used for first-strand cDNA synthesis with

Im-Prom RT (Promega) using a deoxyribosylthymine (dT) primer. Quantitative PCR (qPCR) analysis was performed using SYBR green PCR Master Mix (Applied Biosystem) according to the manufacturer's recommendations in an ABI Prism 7000 (Applied Biosystems). The fragments were amplified with the following primer set: CXCL12 (forward, 5'-TCT GAG AGC TCG CTT GAG TG-3'; reverse, 5'-GTG GAT CGC ATC TAT GCA TG-3') and β -ACT as a reference (forward, 5'-ACA CTG TGC CCA TCT ACG AGG GG-3'; reverse, 5'-TGA TGG AGT TGA AGG TAG TTT CGT GGA T-3'). A standard curve was constructed for each PCR fragment, the reference, and the target. Amplification was monitored in real time and allowed to proceed in the exponential phase until the fluorescent signal reached a significant value (threshold cycle [C_T]).

The formula used for relative quantification was $2^{-\Delta\Delta C_T}$, i.e., the differential expression of a specific gene between samples.

Generation of virus stocks. 293T cells were transfected with calcium phosphate with 10 μ g of the plasmids, and 5×10^5 cells were plated in 6-well tissue culture plates. The culture medium was replaced with fresh DMEM 8 h and 24 h after transfection. The cell supernatants were harvested 48 h after transfection and frozen in aliquots at -80°C . The amounts of p24 viral antigen in the supernatants were quantified using commercially available antigen capture enzyme-linked immunosorbent assay (ELISA) kits (Innotest HIV antigen MAb; Innogenetics).

The transfecting DNA mixture for generating env-pseudotyped lentivirus particles was composed of 10 μ g of psPAX2, 20 μ g of pTRIP-siCXCL12, and 5 μ g of the glycoprotein-encoding plasmid pcDNA3-VSVG.

HIV-1 infection assays. MDDCs (3×10^6 to 5×10^6 per well in a 6-well plate) were incubated with HIV-1 (200 ng of CA-p24) for 2 h at 37°C to allow adsorption of the virus. The cells were then washed in phosphate-buffered saline (PBS) to remove unbound virus (the last wash was negative for p24) and cocultured with activated lymphocytes (5×10^6) in a 6-well plate. MDDCs or activated lymphocytes cultured alone were pulsed with the same amount of HIV-1 (200 ng of CA-p24).

When the infection assays were performed by adding both viruses, JR-CSF and NL4-3, in the same well, MDDCs were incubated for 2 h with identical concentrations of CA-p24 (200 ng of each). Luciferase activity was measured in cell lysates 48 h postinfection. Half of the cells in one well were measured using the *Renilla* Luciferase Assay System kit and the other half with a firefly luciferase substrate (Luciferase Assay System).

To study the roles of *cis* and *trans* mechanisms of HIV-1 transmission, Transwell cell culture plates with 0.4- μ m-pore-size polycarbonate membrane inserts (Corning) were used to separate MDDCs from lymphocytes.

In the neutralization assays with antibodies against CXCL12, MDDCs were incubated with 30 μ g/ml K15C MAb or an equal amount of isotype-matched mouse IgG2a for 30 min at 37°C before being cocultured with activated lymphocytes. In the assays using siRNA expression constructs, MDDCs were transfected as described above, and after 30 min, NL4-3Ren or JRRen viruses were added.

In all the infection assays, cells were collected at 48 h for measuring luciferase activity (relative luciferase units [RLU]) in the cell lysates with a luciferase reporter assay kit using a Sirius luminometer (Berthold Detection Systems).

Immunohistochemical analysis. Staining of the tissue cryosections was performed as described previously (30). Antigen retrieval was performed by heating before immunostaining. Endogenous peroxidase was quenched in 3% H_2O_2 in methanol for 20 min. Staining was performed following a standard indirect avidin-biotin horseradish peroxidase method and developing with diaminobenzidine (Vector Laboratories). Sections were counterstained with hematoxylin.

Immunostaining and confocal microscopy. Cells (MDDCs and Jurkat cells) were washed with ice-cold PBS, followed by fixation in 2% paraformaldehyde and 0.025% glutaraldehyde for 10 min at room temperature. The cells were permeabilized with 0.25% Triton X-100, followed by being blocked with 0.2% BSA for 10 min at room temperature. Slides were incubated with anti-CXCL12 (K15C) or an isotype-matched control (mouse IgG2a) overnight at 4°C . After being washed in PBS containing 0.2% BSA, samples were incubated with Alexa 488 goat anti-mouse antibody (Molecular Probes) at 1:500 for 1 h at room temperature.

CD4^+ T cells negatively enriched from PBMCs by magnetic cell sorting (Miltenyi Biotec) were labeled with the cytoplasmic dye CellTracker green (Molecular Probes).

To facilitate conjugate formation, 5×10^5 effector cells (HIV-1-pulsed mMDDCs) were mixed with an equal number of target cells (activated CD4^+ T cells) at 37°C on poly-L-lysine (Sigma-Aldrich)-treated coverslips for 30 min, with or without the inclusion of MAb K15C. The cells were fixed in 4% formaldehyde in PBS-1% BSA for 15 min at 4°C . For intracellular staining of HIV-1 Gag, the conjugates were permeabilized in 0.1% Triton X-100-5% fetal calf serum (FCS).

Rabbit antisera against HIV-1 Gag p17 and p24 were obtained from the CFAR. Primary antibodies were visualized by single staining using tetramethyl rhodamine isothiocyanate (TRITC)-anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The appropriate controls without primary antibody were performed.

Stained coverslips were analyzed using a Bio-Rad confocal microscope and processed using Metamorph v6 (Universal Imaging Corporation) and Photoshop 7.0 (Adobe Inc.).

RESULTS

CXCL12 production in lymph nodes and cultures of MDDCs. We hypothesized that the inefficient transmission and propagation of X4 HIV strains during early and chronic infection might be due to CXCL12 expression within secondary lymphoid tissue. We therefore analyzed CXCL12 expression within lymph node tissue and determined the cell types producing the chemokine by immunohistochemical analysis using the monoclonal anti-CXCL12 antibody (K15C). As shown in Fig. 1Ai, CXCL12 staining was observed in endothelial cells, in histiocyte-like cells localized in follicular regions, and in parafollicular dendritic cells. Using immunofluorescence techniques (Fig. 1Aii), strong expression of CXCL12 was detected in the paracortical areas of lymph nodes. Interestingly, the large majority of DC-SIGN-positive cells produced CXCL12, demonstrating the expression of this chemokine by DCs.

CXCL12 production in an *in vitro* culture system of MDDCs was assessed by flow cytometry (Fig. 1Bi) and immunofluorescence (Fig. 1Bii). We observed CXCL12 expression in both iMDDCs and mMDDCs, but production was always higher in mature than in immature MDDCs. Also, CXCL12 levels in culture supernatants from MDDCs were quantified by ELISA at 16 pg/ml in iMDDC and 133 pg/ml in mMDDC cultures.

Enhancement of HIV-1 transfer from mMDDCs to target cells is higher for R5 viruses. To analyze the role of DCs in the propagation of R5 and X4 strains, HIV-1-pulsed mMDDCs were cultured with autologous T cells. mMDDCs were pulsed for 2 h with R5 or X4 HIV-1 stocks carrying a luciferase reporter gene using equivalent amounts (200 ng) of Gag p24, washed, and cultured in the presence of activated T cells. The cells were assayed for luciferase activity 48 h later (Fig. 2A). As controls, activated T cells and mMDDCs were challenged separately with virus using equivalent amounts (200 ng) of Gag p24. Infection with an R5 HIV-1 strain (JRRen) was increased by a mean of 7-fold when lymphocytes were cocultured with autologous dendritic cells compared to direct infection of activated lymphocytes with cell-free virus. In contrast, when an X4 HIV-1 strain (NL4-3Ren) was used, no enhancement in T-cell infection by dendritic cell-mediated transfer was observed (0.89-fold compared to cell-free virus infection). No luciferase activity was detected in mMDDCs cultured in the absence of lymphocytes, suggesting that mMDDCs did not become infected under these conditions. Conjugates between mMDDCs pulsed with NL4-3Ren or JRRen and CD4^+ T cells were immunostained with MAbs to Gag and analyzed by laser scanning confocal microscopy (Fig. 2B). Despite detection of similar levels of Gag protein in mMDDCs, Gag labeling in lymphocytes was preferentially observed for R5 viruses (JRRen), while for X4 viruses (NL4-3Ren), Gag protein was infrequently detected.

To analyze the enhancement of R5-tropic viruses in the context of mixed infection with both R5 and X4 viruses and to

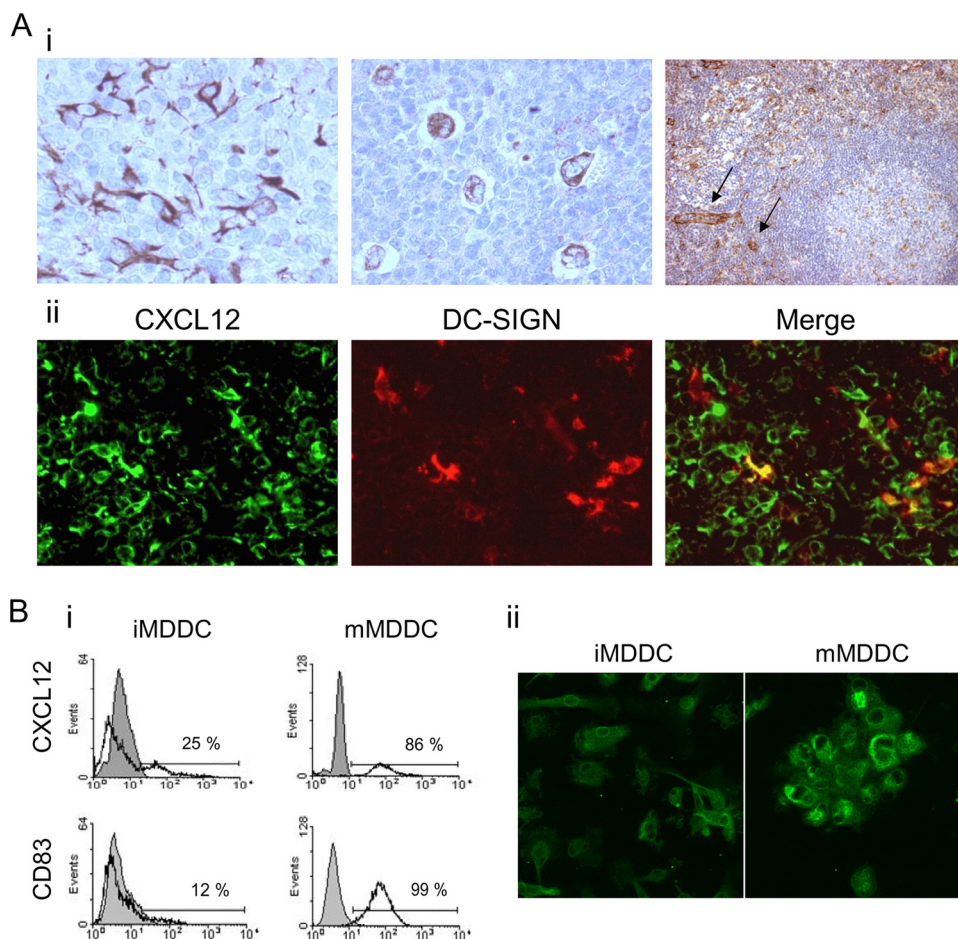


FIG. 1. CXCL12 expression. (A) CXCL12 immunostaining in lymph nodes. (i) Cryosections from normal lymph nodes were examined for the expression of CXCL12 using the K15C specific monoclonal antibody. In the parafollicular zone, there were cells with dendritic morphology positive for CXCL12 staining (left; original magnification, $\times 100$). In germinal centers, cells displaying a histiocyte-like morphology showed CXCL12 immunostaining (middle; original magnification, $\times 100$). Endothelial cells were also immunostained by anti-CXCL12 MAb (arrows in right panel; original magnification, $\times 20$). Sections were counterstained with hematoxylin. (ii) Staining with CXCL12 (left; green) and DC-SIGN (center; red) in lymph nodes using immunofluorescence techniques showed double labeling of DCs (right; merged). (B) Increased CXCL12 expression in mature dendritic cells. (i) Flow cytometry. Cell surface expression of the maturation marker CD83 was determined by direct immunofluorescence and intracellular expression of CXCL12 by indirect immunofluorescence. The percentages of positive cells are shown in the graph. The fluorescence values of appropriate isotype controls are represented by gray filled lines. (ii) CXCL12 expression in iMDDCs and mMDDCs by confocal microscopy. iMDDCs and mMDDCs were plated on poly-L-lysine-coated coverslips, fixed with formaldehyde, permeabilized, and stained with K15C.

simulate conditions where an individual is exposed to both viruses simultaneously, cells were infected with a mixture of R5 and X4 viruses harboring two different luciferase markers (*Renilla* or firefly). Luciferase activity was also measured in cultures infected singly (monoinfection) with each virus using equivalent concentrations of Gag p24. As shown in Fig. 3, the outcomes were very similar when lymphocytes were infected with single or mixed viral preparations. There was a preferential transmission of R5 over X4 viruses when both were present at similar concentrations in T-cell-mMDDC cocultures.

To confirm these results with recombinant viruses carrying envelope glycoproteins from clinical viral isolates, mMDDCs were incubated with recombinant viruses encoding R5 and X4 envelopes amplified directly from patient viral populations (Fig. 4). The profile shown by the infection

with recombinant viruses generated from HIV-1-infected patients was very similar to the one presented by reference R5 and X4 viruses.

X4 HIV-1 transmission from mMDDCs to lymphocytes is lower than from iMDDCs. As higher levels of CXCL12 production were detected in mMDDCs than in iMDDCs (Fig. 1B), we analyzed the enhancement of viral propagation using iMDDCs or mMDDCs pulsed with R5 or X4 viruses (Fig. 5A). In contrast with the absence of infection enhancement observed when lymphocytes were cultured with X4-pulsed mMDDCs, enhancement was observed when lymphocytes were cocultured with X4-pulsed iMDDCs. These data correlate with the levels of CXCL12 production and suggest that CXCL12 produced by mMDDCs interferes with the transmission of X4-tropic strains. As expected, similar levels of enhancement in R5 infection were detected when lymphocytes were cocultured with either iMDDCs or mMDDCs.

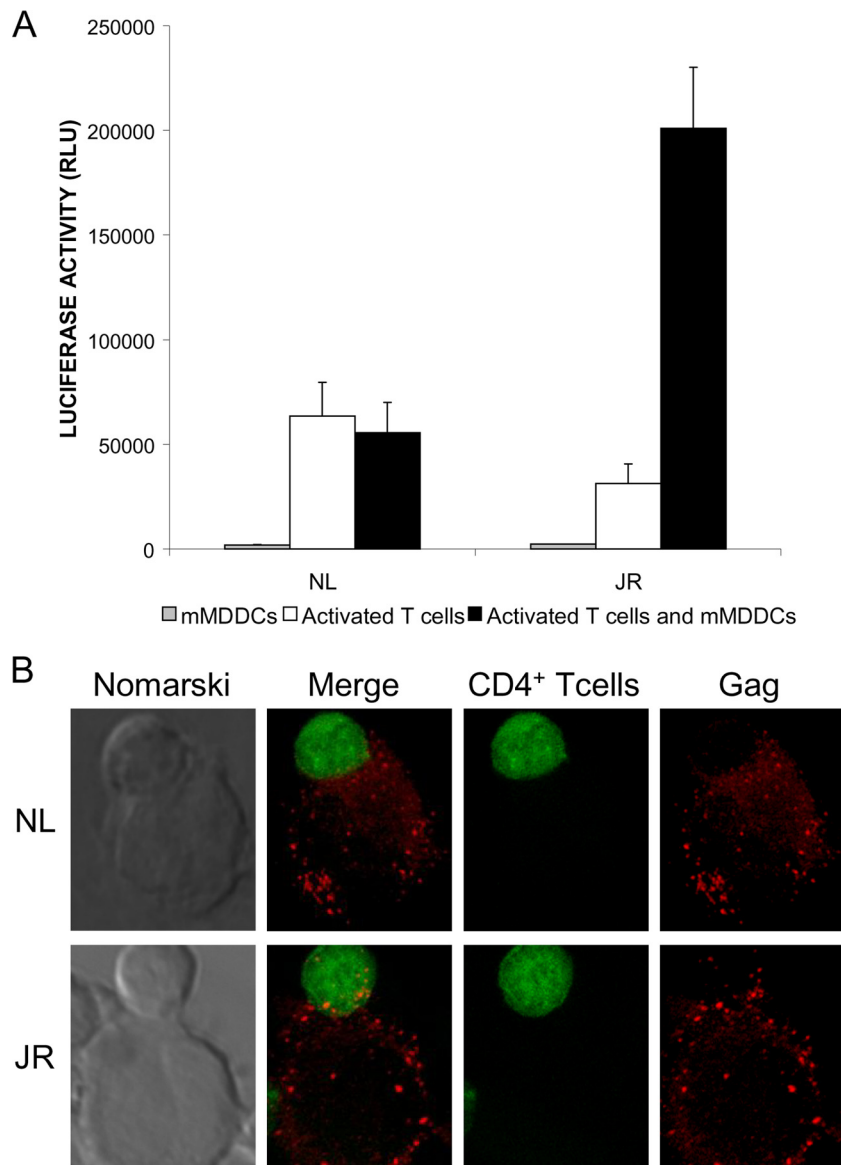


FIG. 2. mMDDCs enhance R5, but not X4, HIV infection. (A) mMDDCs were incubated with NL4-3Ren (NL) (X4 HIV-1) or JRRen (JR) (R5 HIV-1). Activated lymphocytes were infected with NL4-3Ren or JRRen. mMDDCs were pulsed with NL4-3Ren or JRRen, washed with PBS to remove unadsorbed virus, and cocultured with activated lymphocytes. RLU were determined 48 h after infection. Shown are the means and standard errors of the mean (SEM) of 15 experiments. (B) Conjugates between NL4-3Ren- or JRRen-pulsed mMDDCs and CD4⁺ T cells were fixed, permeabilized, and stained by indirect immunofluorescence with a MAb specific for Gag (red). CD4⁺ T cells were prelabeled with a cytoplasmic dye (green). A three-dimensional reconstruction of a z series of images is shown. One representative field out of 20 is shown.

HIV-1 transmission from MDDCs to lymphocytes does not occur in Transwell cultures. To get a better insight into the roles of *cis* and *trans* mechanisms of HIV transmission from dendritic cells to lymphocytes, we performed Transwell assays in which iMDDCs or mMDDCs were seeded in lower wells and T cells were seeded in upper wells for infection assays with X4 and R5 viruses. As shown in Fig. 5B, in the absence of cell-to-cell contact, no infection was detected in T cells seeded in the upper wells. This finding strongly suggests that under the conditions tested, T lymphocytes were infected in *trans* through transfer from noninfected DCs and not in *cis* by virions released from infected dendritic cells.

CXCL12 neutralization enhances X4 HIV-1 infection of lymphocytes cultured with autologous mMDDCs. HIV-1-pulsed mMDDCs were preincubated with antibodies against CXCL12 prior to the addition of lymphocytes. The use of the CXCL12 neutralizing antibody K15C increased infection with X4 viruses 2.4-fold (Fig. 6A). These data indicate that infection with an X4 HIV-1 strain was inhibited when lymphocytes were cocultured with mMDDCs and that this inhibition was dependent on CXCL12 production. In contrast, infection with R5 HIV-1 strains was not modified in this coculture model, and anti-CXCL12 antibody had no effect on infection with R5 viruses.

Finally, we analyzed the effect of CXCL12 on HIV-1 transfer

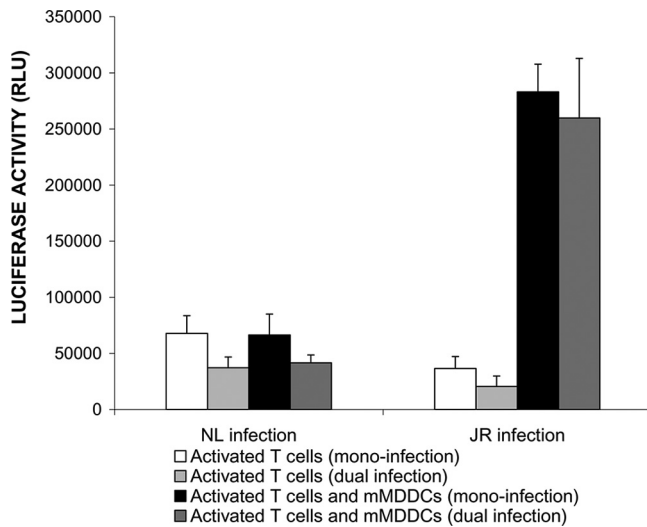


FIG. 3. Coinfection with R5 and X4 viruses. Dual-infection assays involved the addition of the two HIV-1 isolates (JR and NL) at similar concentrations carrying different luciferase reporters, firefly and *Renilla*, and were performed alongside the monoinfections. The graph shows the RLU values in monoinfected cells and in dual-infection assays using an R5 and an X4 virus with different luciferase markers. On the left, measurement of luciferase reflected infection of NL4-3 (X4-tropic virus) under different conditions (mono- or dual infection). On the right, the luciferase levels indicated infection with the JR-CSF strain (R5-tropic virus). Shown are the means and SEM of four experiments.

by laser scanning confocal microscopy. For this, we established a system based on the formation of conjugates between mMDDCs pulsed with NL4-3Ren virus and CD4⁺ T cells. Such conjugates were formed at 37°C in the presence or absence of the antibody K15C, and conjugates were examined for viral Gag localization. When the antibody against CXCL12 was added, HIV-1 was often detected in the conjugated T cells,

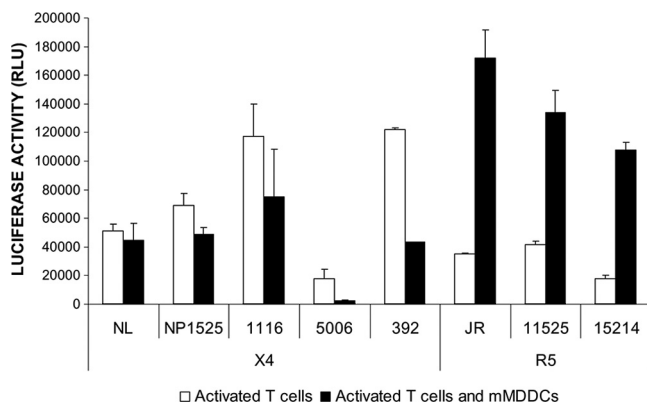


FIG. 4. mMDDCs enhance infection of R5, but not X4, HIV-1 isolates carrying the envelope from patients' isolates. Lymphocytes were activated with PHA and IL-2 for 7 days and infected with recombinant viruses carrying Env from HIV-infected patients (white bars). mMDDCs were pulsed with these viruses, washed with PBS to remove unadsorbed virus, and cocultured with activated lymphocytes (black bars). RLU were determined 48 h after infection. JR, 11525, and 15214 are R5 viruses. NL, NP1525, 1116, 5006, and 392 are pure X4 viruses. Shown are the means and SEM of three experiments.

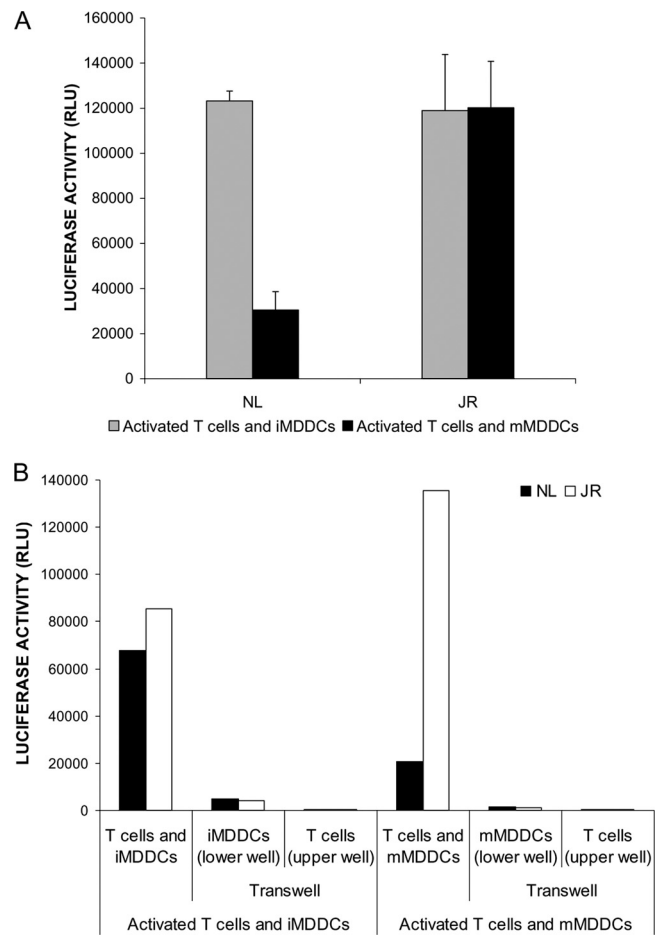


FIG. 5. (A) Increased transmission of X4 strains to lymphocytes is produced by iMDDCs, but not by mMDDCs. Infection of lymphocytes cocultured with iMDDCs is represented by gray bars. The black bars correspond to the infection of lymphocytes cocultured with mMDDCs. Shown are the means and SEM of three experiments. (B) HIV-1 transmission from MDDCs to lymphocytes does not occur in Transwell cultures. mMDDCs or iMDDCs were incubated with NL4-3Ren or JRren, washed, and cocultured with activated lymphocytes in the same well (T cells and i/mMDDCs) or separated by a polycarbonate membrane (Transwell). When the Transwell membrane was present, cell lysates were obtained from both the upper compartment (T cells) and the lower well (MDDCs). RLU were determined 48 h after infection. One experiment out of two is shown.

showing transfer of X4 virus to lymphocytes (Fig. 6B). On the other hand, the Gag protein detection levels in T cells did not increase for R5 viruses when the antibody K15C was added (Fig. 6B).

CXCL12 siRNAs enhance X4 HIV-1 infection of lymphocytes cultured with autologous mMDDCs. With the aim of silencing human CXCL12 expression, we designed siRNAs targeting the CXCL12 gene (Table 1). The capacity of siRNAs to inhibit CXCL12 expression was tested by cotransfection of Jurkat cells with a control plasmid (pSUPER-siGFP) or pSUPER-siCXCL12 constructs (pSUPER-siCXCL12 94, pSUPER-siCXCL12 183, and pSUPER-siCXCL12 244), together with a human CXCL12 expression plasmid. Cells were stained with the anti-CXCL12 K15C MAb, and protein expression was analyzed by immunofluorescence (Fig. 7A). Furthermore, siRNA

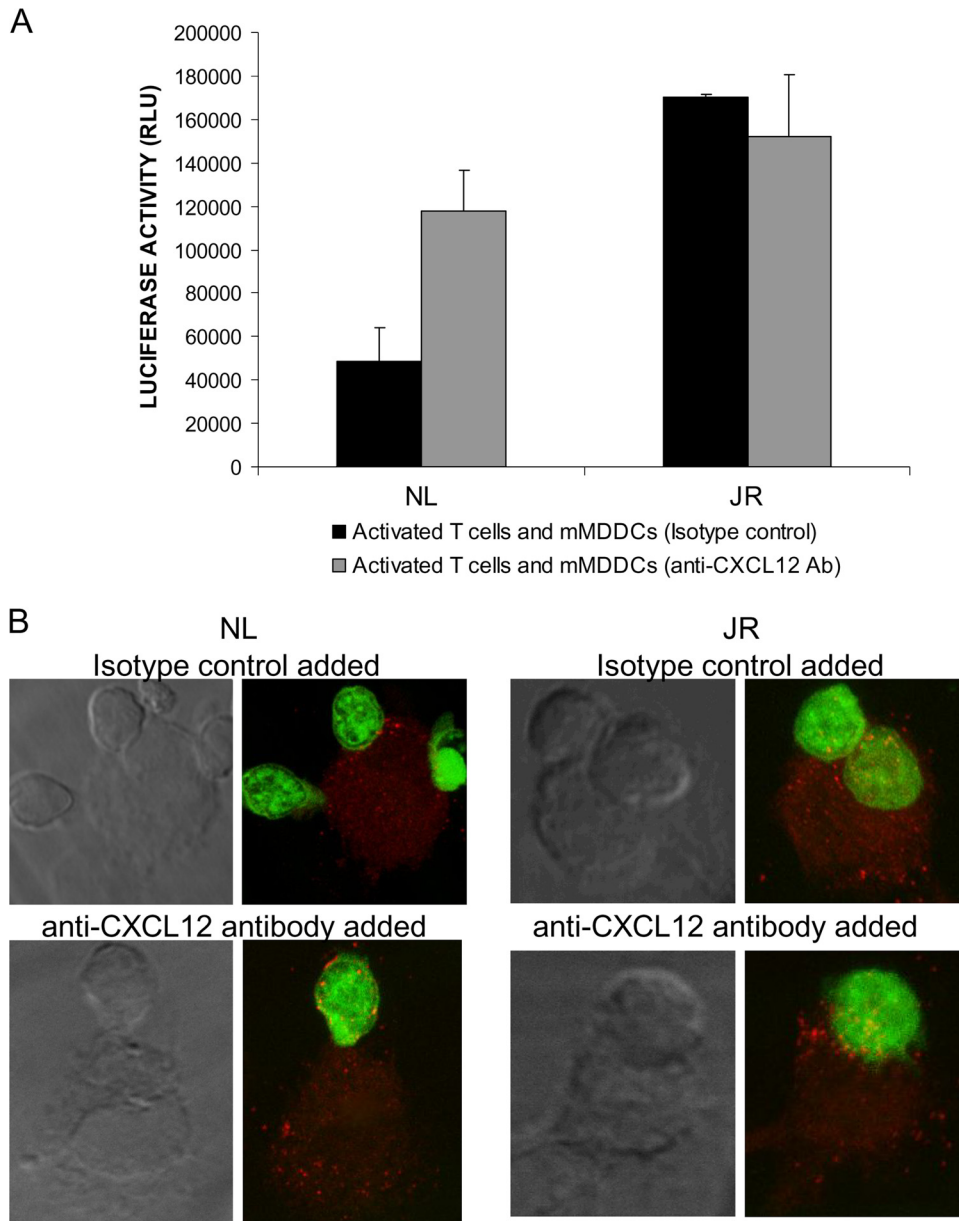


FIG. 6. Blocking of the CXCL12 effect by the anti-CXCL12 antibody K15C increased infection of HIV-1 X4 strains. (A) mMDDCs were pulsed for 2 h with NL4-3Ren or JRRen, and unbound virus particles were washed away. Then, the mMDDCs were preincubated with the neutralizing antibody (Ab) K15C (gray bars) or an isotype control (black bars) for 30 min at 37°C and cocultured with activated lymphocytes. Shown are the averages and SEM of three experiments. (B) Conjugates were formed between NL4-3Ren- or JRRen-pulsed mMDDCs and CD4⁺ T cells in the absence or presence of K15C. The cells were fixed, permeabilized, and stained for Gag (red). CD4⁺ T cells were prelabeled with a cytoplasmic dye (green).

knockdown of CXCL12 expression was confirmed by qPCR. As observed in Fig. 7B, transduction of MT-2 cells, which express CXCL12 naturally, with CXCL12 siRNA-expressing lentiviruses provoked a significant decrease in the expression of the chemokine compared to cells infected with control siRNA-expressing lentivirus. The ability of siRNAs to inhibit CXCL12 expression in mMDDCs was confirmed by immunofluorescence (Fig. 7C).

We studied the effects of CXCL12-specific siRNAs on HIV-1 *trans*-enhancement of activated T cells. For this purpose, mMDDCs were transfected with the control plasmid

pSUPER-siGFP or with the three pSUPER-siCXCL12 constructs (pSUPER-siCXCL12 94, pSUPER-siCXCL12 183, and pSUPER-siCXCL12 244). The efficiency of the transfection of mMDDCs (25%) was measured by transfecting these cells with the plasmid pmaxGFP and analysis by flow cytometry (Fig. 7D). After 30 min, JRRen or NL4-3Ren was added and incubated for 2 h before the addition of lymphocytes. mMDDCs transfected with the mixture of pSUPER-siCXCL12 vectors were 3-fold more efficient at transferring NL4-3Ren to activated lymphocytes than mMDDCs transfected with the control vector (Fig. 7D). This increase in in-

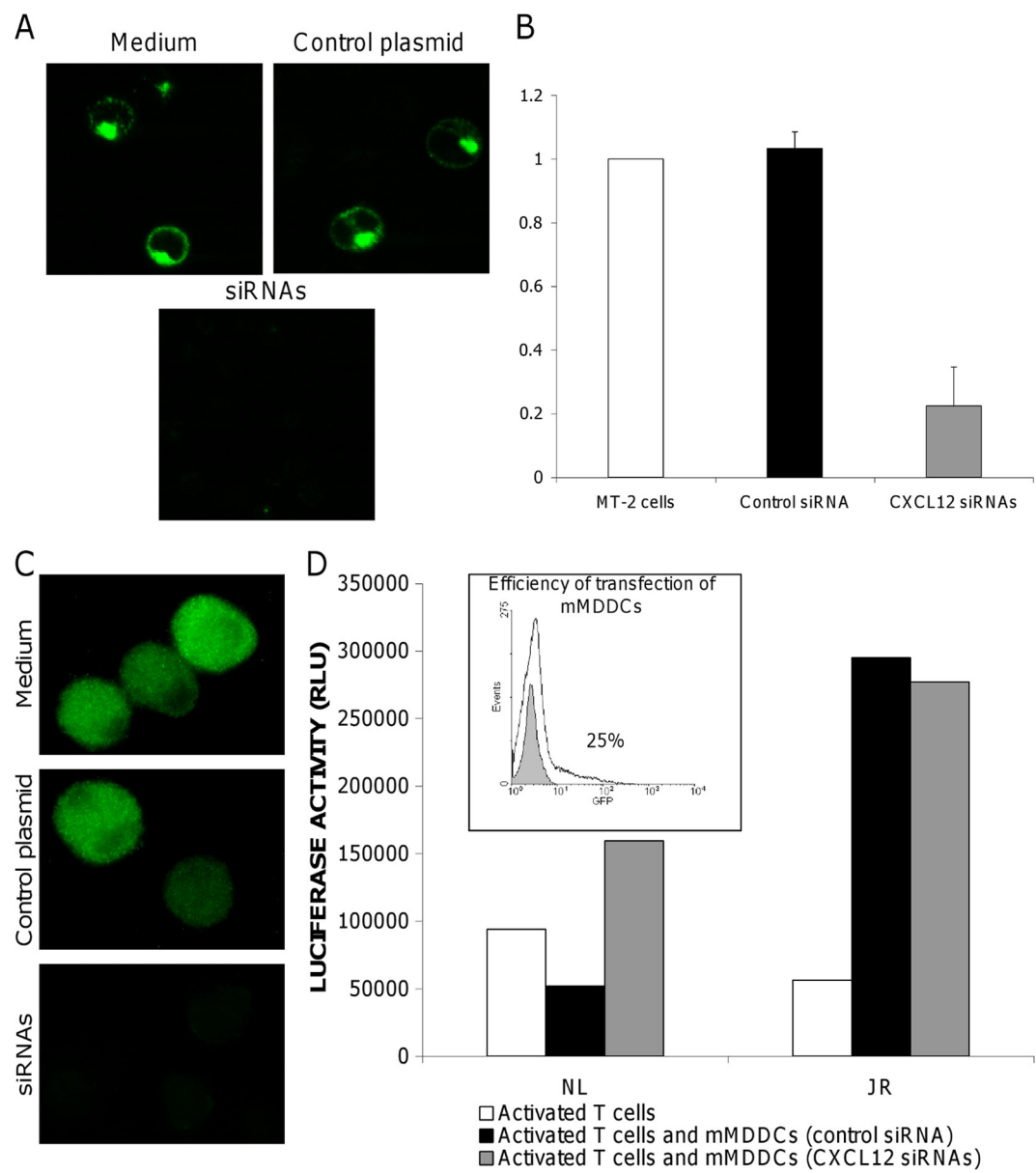


FIG. 7. siRNA directed against CXCL12 increased infection by HIV-1 X4 strains. (A) CXCL12 expression in Jurkat cells that were untransfected or transfected with a plasmid control or with siRNA expression constructs against CXCL12. (B) qPCR analysis of CXCL12 mRNA expression in MT-2 cells that were uninfected (white bars) or infected with control siRNA-expressing lentiviruses (black bars) or with CXCL12 siRNA-expressing lentiviruses (gray bars). Shown are the means and SEM from two experiments. (C) CXCL12 expression in mMDDCs that were untransfected or transfected with a plasmid control or with siRNA expression plasmids against CXCL12. (D) mMDDCs were transfected with a plasmid control (black bars) or with siRNA expression vectors against CXCL12 (gray bars), pulsed with NL4-3Ren or JRren, and incubated with lymphocytes. The direct infection of lymphocytes is represented by white bars. One representative experiment out of three is shown. The inset shows the efficiency of transfection of mMDDCs with pmaxGFP.

fection did not occur in mMDDCs transfected with pSUPER-siCXCL12 constructs and pulsed with the virus JRren compared to those transfected with the control plasmid. These data confirmed that CXCL12 produced by mMDDCs interfered with the transmission of X4 strains.

DISCUSSION

DCs capture HIV-1 at mucosal entry sites and transport virus to the T-cell compartment in lymphoid tissues, where

DC-associated HIV-1 is efficiently transmitted to CD4⁺ T cells (10, 33). HIV-1 specifically binds to DCs through the interaction of gp120 with DC-SIGN (17) or other lectin-like receptors (42). Once present within lymph nodes, HIV-1 may pass directly from DCs to T cells in the parafollicular area or may become trapped on follicular dendritic cells in lymphoid follicles.

The observation that transmission of infection is confined to R5 strains of HIV-1 has remained an enigma. The selective R5 HIV-1 transmission via sexual intercourse could be caused by

CXCL12 production by cells at mucosal surfaces, which may reduce the transmission and propagation of X4 HIV-1 at these sites (1). This reduced transmission of X4 HIV-1 correlates with the predominantly intracellular expression of CXCR4 in resident skin Langerhans cells (49) and intestinal intraepithelial lymphocytes (1). CXCL12 protein and mRNA expression have also been observed in endothelial cells, pericytes, and CD1a⁺ dendritic cells (30). However, these findings do not explain why R5 isolates are also selected in primary infection via the intravenous route or the persistence of R5-tropic strains for years in HIV-1-infected patients. The latter observation is particularly difficult to understand, since relatively few mutations in the highly variable V3 loop can account for a switch of HIV-1 coreceptor use from CCR5 to CXCR4 (38, 46). Despite the fact that in some particular contexts, such as treatment with CCR5 antagonists, multiple changes in the receptor do not have as a consequence a switch to CXCR4 use (25), *in vivo* the earliest detectable X4 variants show only one or two amino acid substitutions compared to coexisting R5 variants (22). These observations point to the existence of selective pressure against X4 HIV-1 evolution, the exact nature of which remains to be established. A role for humoral immunity in HIV-1 tropism evolution has been proposed, and in some patients, the increased neutralization of recently emerged X4 strains can potentially contribute to the late emergence of X4 variants. However, in other patients, the absence of neutralizing antibodies against X4 viruses suggests that other selective pressures are also involved (8).

Based upon the data presented in the current study, and particularly CXCL12 production by DC-SIGN-positive DCs in the parafollicular T-cell areas of lymph nodes, we propose that CXCL12 production at the DC-T-cell infectious synapse (31) is a selective pressure interfering with X4 HIV-1 use of CXCR4 and thereby the emergence and propagation of X4 HIV-1 strains *in vivo*. In further support of this proposal, we demonstrated high expression levels of CXCL12 by a variety of cell types, mainly endothelial and stromal cells in the parafollicular T-cell area in lymph nodes. CXCL12 production by these cell types produces a general tissue environment containing high local CXCL12 levels that would not favor the propagation of X4 viruses.

According to this hypothesis, a decrease in CXCL12 production secondary to the destruction of lymph node architecture could contribute, together with other mechanisms of immune failure, to the emergence of viruses using the CXCR4 receptor in advanced stages of HIV disease. As an alternative mechanism, the switch from R5- to R5X4- or X4-tropic viruses could be related to the generation of viral envelopes displaying high avidity for CXCR4 that would compete efficiently with CXCL12 binding to the receptor.

Using an *in vitro* infection system of lymphocytes cocultured with mMDDCs previously pulsed with HIV-1, we observed that transmission and propagation of X4 viruses was worsened in comparison with R5 strains. Interestingly, the inhibitory effect provided by mMDDCs was even stronger for viral chimeras carrying the viral envelope from HIV-infected patients than for the reference laboratory-adapted strain NL4-3. Overall, these results confirm the lack of *trans*-enhancement for X4 viruses in the infectious synapse, in contrast with R5-tropic strains. This difference between R5 and X4 virus infections was

not due to higher levels of CCR5 expression on target lymphocytes, as described by other authors (20). Actually, after treatment with PHA and IL-2, CXCR4 was expressed in 80 to 90% of lymphocytes, whereas CCR5 expression was detected in 20 to 30% (data not shown). It has been reported that intracellular expression of Nef protein from HIV-1 and other lentiviruses downregulates CXCR4 from the cell membrane (29, 45). The recombinant viruses used do not express Nef, and therefore, the effect is not relevant to this experimental system. However, it can be argued that in the presence of Nef decreased infection of lymphocytes in the infectious synapse could be related to CXCR4 downregulation secondary to Nef expression and not to an inhibitory effect of CXCL12. However, CXCR4 downregulation occurs in infected cells expressing Nef, and therefore, at least for the first round of infection, T lymphocytes would be susceptible to infection with CXCR4 strains. Furthermore, downregulation induced by Nef has been reported, not only for CXCR4, but also for CCR5 and other chemokine receptors. The different patterns of R5 and X4 transmission observed in the dendritic cell-lymphocyte environment cannot be attributed to this mechanism.

The worst transmission of X4 viruses was related to CXCL12 production. By blocking studies with CXCL12 neutralizing antibody or by siRNA knockdown of CXCL12 expression in DCs, X4 virus *trans*-enhancement was mediated in DC-T-cell conjugates. In contrast, the *trans*-enhancement of R5 virus infection from DCs to T cells was predictably not affected by the high levels of CXCL12 expression, and this was the case even when R5 virus was introduced in the presence of an equivalent amount of X4 virus.

Yamamoto et al. (48) observed selective replication of R5 over X4 HIV-1 isolates in CD4⁺ T cells cultured with iMDDCs. This increased infectivity was dependent on the activation state of CD4⁺ T cells, since weakly activated CD4⁺ T cells were preferentially infected by R5 viruses. Our results agree with those of Yamamoto et al., as we observed that in a coculture of iMDDCs and PHA-stimulated T cells, both R5 and X4 viruses were able to replicate at similar levels. In contrast, we found preferential replication of R5 viruses in PHA-activated lymphocytes when they were cocultured with mMDDCs. Therefore, the activation state of CD4⁺ T cells would not be the only factor implicated in R5 selection in the infectious synapse.

Previous studies have found an enhancement of both X4 and R5 viruses mediated by DC-SIGN. However, in these experiments, DC-SIGN-expressing immortalized cell lines (17, 41) or immature dendritic cells (14, 23, 32, 41) were used, which either did not express CXCL12 or produced only small amounts of the chemokine. As in previously published studies, in our *in vitro* system, when lymphocytes were cocultured with iMDDCs, *trans*-enhancement of HIV-1 infection was observed both when R5 HIV-1 was used and when X4 HIV-1 was used. Other studies have described increased transmission of X4 HIV-1 by mature dendritic cells (26, 35). Differences in the stimuli employed for MDDC maturation and the use of cell lines or purified naïve lymphocytes as target cells could account for this discrepancy (26, 35). Also, higher (between 10 and 50 times) viral inputs were used in those experiments than in our experimental model (26, 35), which could potentially

overwhelm the blocking capacity of CXCL12 produced by dendritic cells.

A two-phase transfer of HIV-1 from MDDCs to lymphocytes has been previously described elsewhere as follows: an early short-term phase of viral transfer *in trans*, caused by the direct binding of viral particles to DC-SIGN or other molecules at the surfaces of the MDDCs, and then a later long-term transfer phase (from 48 h to 72 h after viral exposure) that depends on the ability of MDDCs to complete *de novo* viral production (9, 43). However, for our lymphocyte-MDDC co-culture model's HIV-1 exposure of less than 48 h, HIV-1 transfer to lymphocytes secondary to MDDC infections probably does not take place, which is suggested by the lack of luciferase activity detection in our pure MDDC cultures. The results from Transwell experiments strongly suggest that under the conditions tested, lymphocytes were infected *in trans* through transfer from noninfected MDDCs and not *in cis* by virions released from productively infected dendritic cells. Nevertheless, CXCL12 production by mMDDCs could selectively interfere with the transmission of X4 HIV-1 isolates to neighboring CD4 T lymphocytes during both the early and later phases after viral exposure.

In summary, we propose that during the process of maturation of dendritic cells, the expression of the chemokine CXCL12 is upregulated. As a consequence, increased CXCL12 production by mDCs could naturally and selectively block the transmission of X4 HIV-1 strains after HIV-1 exposure. This barrier could partly explain the lack of X4 virus detection during early and chronic HIV-1 infection.

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